Reduced Somaclonal Variation in Barley Is Associated with Culturing Highly Differentiated, Meristematic Tissues

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ABSTRACT

Modern plant breeding programs depend heavily on germplasm resources composed of closely related breeding lines and cultivars. Asexual introduction of recombinant DNA offers novel opportunities for crop improvement, but most transformation methods rely on tissue culture systems which are mutagenic. The resultant transgenic plants frequently contain undesirable genetic changes (somaclonal variation), in addition to the introduced transgene. Such plants may have reduced agronomic performance, which complicates their use as parents in a breeding program. The development of tissue culture systems that are less mutagenic should enable the production of transgenic plants with superior performance. In this study, agronomic traits were measured for plants regenerated from cultures of two barley (*Hordeum vulgare* L.) genotypes, using three different tissue culture systems, and compared with the performance of uncultured controls. Plants derived from all three systems were shown to have reduced performance for one or more agronomic traits, but there were clear differences attributable to the culture system. Plants derived from standard embryogenic callus tissues were shown to have the greatest reductions in agronomic performance. Two other tissue culture systems, which had been developed for increased regenerability, showed better performance. Plants derived from highly differentiated, meristematic tissues showed the least reductions in agronomic performance. Plants derived from a modification of the embryogenic callus system—which is characterized by an intermediate level of differentiation—showed intermediate levels of agronomic performance. These results demonstrate that modifications of methodology, in addition to improving plant regenerability, can reduce the level of somaclonal variation in regenerated plants.

Modern, elite crop germplasm has been created through selective breeding by assembling particular allelic packages which interact to produce desired agronomic and quality traits. Disturbance of *successful genomes* by hybridization with unadapted or *wild* germplasm, or by introducing genetic and epigenetic variability in vitro, is undesirable because it will slow subsequent cultivar development. To create transgenic germplasm most useful to the breeder, asexual genetic manipulations should result only in the specific and limited changes caused by the expression of the introduced sequences.

All widely used techniques for asexually manipulating barley require DNA delivery to cells grown in vitro (Wan and Lemaux, 1994; Jähne et al., 1994; Tingay et al., 1997; Kihara et al., 1998). Undesirable, tissue culture-induced variation, or somaclonal variation (SCV; Larkin and Skowcroft, 1981), can accompany the intentional changes in regenerated plants. SCV is associated with significant and heritable genetic and epigenetic changes that are widespread throughout the genome (Karp, 1994; Olhoft and Phillips, 1999; Kaeppler et al., 2000).

In nontransgenic barley, SCV can result in severe reductions of agronomic performance and grain quality (Bregitzer and Poulson, 1995; Bregitzer et al., 1995; Kihara et al., 1996), although other studies have shown only minor impacts of somaclonal variation (Baillie et al., 1992). Of greater importance, however, are indications that SCV is greatly exacerbated in transgenic barley plants, presumably a consequence of additional genomic stresses imposed during the transformation process (Bregitzer et al., 1998; Choi et al., 2000a,b). Osmotic treatment and selection with either bialophos or hygromycin B has been shown to trigger extensive cytological aberrations in transgenic barley plants (Choi et al., 2001).

Difficulties associated with regeneration of plants from transformed barley cells, and observations of high levels of SCV in regenerated barley plants, led to the development of improved transformation systems for barley (Cho et al., 1998; Zhang et al., 1999; reviewed in Lemaux et al., 1999). Cho et al. (1998) manipulated cytokinin and copper levels to produce a modified embryogenic callus (MEC), or highly regenerative green tissues, that initially is similar in appearance and growth rate to the standard embryogenic callus (SEC) produced by Wan and Lemaux (1994), but which shows a progressive increase in the degree of differentiation in response to increases in the ration of cytokinin to auxin. Zhang et al. (1999) developed a transformation system based on DNA introduction into cells of shoot meristematic cultures (SMCs). Essentially, SMCs are composed of highly differentiated, green meristematic tissues derived from seedling axillary meristems; plant regeneration occurs via organogenesis. The initial development of SMC tissues is slow relative to SEC and MEC tissues, but once established SMC tissues appear similar to SEC and MEC tissues with respect to the rate of tissue proliferation. Both MEC and SMC tissues are characterized by very long-term and high levels of regenerability, relative to SEC tissues. MEC tissues are morphologically intermediate to SEC and SMC tissues, as demonstrated both by visual observation and by as demonstrated in tissues by expression of KN1, a shoot meristem marker (Zhang et al., 1999; Lemaux et al., 1999).

The improved regenerability characteristics of MEC and SMC tissues may be indicative of greater genomic stability relative to SEC tissue. The studies reported

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Abbreviations: MEC, modified embryogenic; SEC, standard embryogenic callus; SCV, somaclonal variation; SMCs, shoot meristematic cultures.

here were conducted to examine the level of SCV in plants derived from these three types of cultured barley tissues, relative to parental controls, as measured by agronomic performance.

MATERIALS AND METHODS

Two cultivars, Golden Promise and Morex, were chosen for study. Golden Promise is highly regenerable but inferior to Morex for agronomic and quality traits important to North American producers and users. Morex exhibits poor regenerability, although heterogeneity exists for this trait in Morex (Bregitzer et al., 2000). Single plant derived lines were developed from Golden Promise and Morex to remove varietal heterogeneity as a potential source of variability for agronomic performance. For each cultivar, a single donor plant was used as the source of all explants; each of these plants was grown, side by side, in the same growth chamber to alleviate or remove the potential confounding effects of differing donor plant environments.

Cultures were established and plants regenerated from these genetically homogeneous lines following published procedures for SEC (Wan and Lemaux, 1994), MEC (DBC1 medium for the first month, DBC2 medium thereafter; Cho et al., 1998), and SMC (Zhang et al., 1999) tissues. SEC and MEC tissues were derived directly from scutellar tissues of immature embryos; SMC tissues were derived from axillary meristems of in vitro-germinated immature embryos. Conditions for the establishment of the three types of tissues were held as constant as possible, except that culture establishment for SEC and MEC tissues was in the dark, and commenced immediately upon embryo placement on initiation medium. In contrast, the establishment of SMC tissues took place under moderate light conditions and did not commence until seedling tissues were available, isolated, and placed on initiation media. Plants were regenerated from all cultures about 1 mo and 3 mo after culture initiation, except that for SMC cultures of Golden Promise plants were regenerated about 3 mo and 6 mo after initiation. All regenerated plants were moved to a single greenhouse at the Berkeley, CA, location for the development of R_1 seed. At approximately the same time, in the same greenhouse, seed was produced from control (seed-derived) plants of Morex and Golden Promise. Variances in growth environment were thus minimal for a given method–regeneration date; plants regenerated after different times in culture necessarily were moved to the greenhouse at different times.

For each regenerated lines, R_2 seed was produced from multiple (approximately 5) R_1 plants, all planted on the same date, in a single greenhouse at the Aberdeen location. Under the same conditions, additional seed was produced of the Morex and Golden Promise control lines. The quantities of seed obtained were limited, but sufficient to enable replicated field tests at two locations. Each genotype–method–regeneration date combination was represented by seven to nine R_1-derived R_2 lines, except for Morex-derived SEC cultures (two and four lines from 1- and 3-mo-old cultures, respectively), which is a reflection of the inherent regenerability problems of Morex. Three replicates were planted in 1997 at Aberdeen (irrigated; 1370 m above sea level) and Tetonia, ID (dryland; 1820 m above sea level). In each replicate, regenerated lines and parental controls were represented by eight plants planted in a single row. Rows consisted of alternating single barley plants and oat (cultivar Ajay) hills consisting of 10 or more plants, spaced approximately 30 cm apart. Rows were staggered internally in such a way that each barley plant was surrounded by four oat hills. This planting arrangement provided a more consistent competitive effect for each barley plant, regardless of the relative competitive vigor of neighboring barley plants or lack of germination of some barley seeds. To further minimize experimental variability, Golden Promise-derived lines and Morex-derived lines were planted in separate blocks within each replicate and treated as separate experiments. R_1 and control plants were harvested individually; for each plant, data were recorded for heading date, height, seed yield, and seed size (as measured by the weight of 100 kernels).

From each regenerated line tested in 1997, three plants were selected randomly and advanced to produce three sublines for the 1998 field trials. R_2 seed from the three selected plants was increased in bulk-harvested rows in New Zealand during the 1997-1998. Six replicates of R_2-derived R_4 seed were planted in 1998 at Aberdeen and at Tetonia. In each replicate, regenerated lines and parental controls were represented by single rows 1.2 m in length. Golden Promise-derived and Morex-derived lines were planted in separate blocks within each replicate to minimize experimental variability. Data were recorded for heading date, height, seed yield, test weight, and the percentage of plump kernels (those retained on a 2.38- $\times$ 19-mm sieve).

Analysis of variance was conducted utilizing PROC GLM within the software provided by SAS (SAS/STAT User's Guide, Version 7.1, SAS Institute, Inc., Cary, NC). All sources of variance were considered to be random, and included location, replicates within locations, genotype, culture method, regeneration date, and interactions between location, genotype, culture method, and regeneration date. Age of culture at the time of regeneration was treated as a qualitative variable (1st date, 2nd date). Data for Golden Promise and derived lines or sublines, and Morex and derived lines or sublines, were analyzed separately for each test year. Findings of significant differences ($P = 0.05$) among methods used to produce the tested materials (self-pollinated controls, SEC-, MEC-, and SMC-derived lines) were further examined. Dunnett's tests were used to identify differences from control performance, and Tukeys's tests were used to identify performance differences among the three culture systems.

RESULTS

General Observations

Growth conditions were generally favorable for both locations in both years, except that nonrandomly distributed field variability forced the exclusion from analysis of one replicate at Aberdeen in 1997, and one replicate at Tetonia in 1998. Of the eight seeds planted per plot in 1997, an average of 5.4 and 7.1 plants germinated at the Aberdeen and Tetonia test locations, respectively. There were no differences in germination rates between controls and regenerated lines. Variability among plants within certain regenerated lines and between certain lines for relative growth rate and tillering capacity was obvious on visual inspection, but no data was collected on these traits. Qualitative mutations were observed, including albino plants, plants with albino streaks, and yellow-green plants; and plants exhibiting extremely poor, stunted growth. These mutations were observed at a low percentage (less than 1%), and no relationship was detected between frequency of mutant plants and culture methods (data not shown).

With two exceptions, random selection of plants in

1997 to provide seed for 1998 tests eliminated chlorophyll and extremely poor-growth mutations. The exceptions were two of the three sublines derived from a single line regenerated from 3-mo-old GP-derived SMC tissues. These sublines were yellow-green in color, significantly shorter, and had lower grain yield, test weight, and percentage plump kernels than the third subline which had normal green color (data not shown). These data were included in the overall analyses presented in this report. Another mutant phenotype that had not been noted in 1997 was discovered in lines derived from two plants selected from 6-mo-old GP-derived SMC tissues. These lines had extraordinarily brittle straw, resulting in extreme culm breakage and the loss of many of the heads well before physiological maturity. During harvesting operations, the majority of the remaining heads were lost. For these lines, data were not recorded for grain yield, test weight, and percentage plump kernels. This phenotype likely was present in the 1997 trials but unnoticed because of the relatively gentle nature of hand harvesting single plants (1997) versus the machine binding of rows prior to threshing (1998).

Heading Date

Although there was a tendency for regenerated plants to head later than the uncultured control (data not shown), analysis of variance did not detect significant differences ($P = 0.05$) among the systems used to produce the tested plants (self-pollination, SEC, MEC, and SMC).

Plant Height

Analysis of variance of data for plant height collected in 1997 (Table 1) showed significant differences attributable to method. The mean heights of regenerated lines were shorter than the uncultured control, but Dunnet's analysis detected significant ($P = 0.05$) differences only for Morex lines derived from SEC tissues at the first regeneration date, and for Morex lines derived from MEC tissues at the second regeneration date.

Data collected in 1998 (Table 2) also identified significant variation attributable to the method of plant production. As in 1997, there was a tendency of regenerated plants to be shorter than the uncultured controls. Dunnett's analysis detected significant differences from the control most often for SEC-derived plants, and least often from SMC-derived plants.

Dunnett's analyses are intended primarily to detect differences from control performance. A more powerful comparison of the effect of culture method on the height of regenerated plants was provided by Tukey's analyses. These analyses (Tables 1 and 2) showed SEC-derived plants were generally shorter than MEC- and SMC-derived plants.

Table 1. Mean height (in centimeters) of lines derived from three culture methods and uncultured parental control plants grown in 1997 at Aberdeen and Tetonia, ID.

	Golden Promise-derived lines		Morex-derived lines	
	Regeneration date		Regeneration date	
Culture Method	1st	2nd	1st	2nd
Control	62.3	62.3	92.0	92.0
SEC	60.8B†	59.3A	86.4*B	87.6B
MEC	62.0A	59.5A	89.9A	86.6*B
SMC	62.9A	60.6A	89.9A	90.9A

* Indicates significant difference ($P = 0.05$) from control performance, as determined by the Dunnett's test.
† Comparisons between SEC, MEC, and SMC: means followed by the same letter are not significantly different ($P = 0.05$), as determined by Tukey's means separation procedure.

Table 2. Mean height (in centimeters) of lines derived from three culture methods and uncultured parental controls grown in 1998 at Aberdeen and Tetonia, ID.

	Golden Promise-derived lines		Morex-derived lines	
	Regeneration date		Regeneration date	
Culture Method	1st	2nd	1st	2nd
Control	64.4	83.2/49.4‡	99.6	99.6
SEC	62.9B†	77.3*/47.9B	93.2*B	93.2*C
MEC	64.1A	78.4*/49.1A	96.9A	95.8*B
SMC	64.0A	77.5*/49.6A	97.5A	98.1A

* Indicates significant difference ($P = 0.05$) from control performance, as determined by the Dunnett's test.
† Comparisons between SEC, MEC, and SMC: means followed by the same letter are not significantly different ($P = 0.05$), as determined by Tukey's means separation procedure.
‡ Method*Location interaction was significant ($P = 0.05$); data presented separately for Aberdeen/Tetonia.

Grain Yield

Tests conducted in 1997 (Table 3) and in 1998 (Table 4) showed regenerated lines to be generally lower yielding than their controls. Significant differences attributable to the method used to produce tested lines were found. Dunnett's analysis of data collected in 1997 showed differences from the control for GP-derived lines from all method–regeneration date combinations except for SMC-derived lines from the first regeneration date, but for Morex-derived lines only those from the second regeneration date showed differences from the control, and these differences were detected only at the Aberdeen test location. In 1998, significant reductions relative to the control were detected only for SEC-derived lines. As in 1997, significant interactions were

Table 3. Mean grain yield (in grams per plant) of tissue culture-derived lines and uncultured parental controls grown in 1997 at Aberdeen and Tetonia, ID.

	Golden Promise-derived lines		Morex-derived lines	
	Regeneration date		Regeneration date	
Culture Method	1st	2nd	1st	2nd
Control	41.3	41.3	47.4	60.6/38.5‡
SEC	29.6*B†	23.5*B	44.0A	42.5*A/36.4A
MEC	33.4*B	26.8*AB	44.4A	44.2*A/34.5A
SMC	37.8A	30.8*A	40.6B	47.7*A/33.8A

* Indicates significant difference ($P = 0.05$) from control performance, as determined by the Dunnett's test.
† Comparisons between SEC, MEC, and SMC: means followed by the same letter are not significantly different ($P = 0.05$), as determined by Tukey's means separation procedure.
‡ Method*Location interaction was significant ($P = 0.05$); data presented separately for Aberdeen/Tetonia.

Table 4. Mean grain yield (in grams per plot) of lines derived from three culture methods and uncultured parental controls grown in 1998 at Aberdeen and Tetonia, ID.

	Golden Promise-derived lines		Morex-derived lines	
	Regeneration date		Regeneration date	
Culture Method	1st	2nd	1st	2nd
Control	266	266	341/182‡	341/182‡
SEC	252B†	220*C	260*C/170A	271*B/166A
MEC	262AB	234B	297B/166A	315A/166A
SMC	266A	252A	317A/170A	320A/175A

* Indicates significant difference (P = 0.05) from control performance, as determined by the Dunnett's test.
† Comparisons between SEC, MEC, and SMC: means followed by the same letter are not significantly different (P = 0.05), as determined by Tukey's means separation procedure.
‡ Method*Location interaction was significant (P = 0.05); data presented separately for Aberdeen/Tetonia.

detected between culture method and test environments for Morex-derived lines, and again these interactions showed greater reductions in yield at the Aberdeen test location. There appeared to be an interaction between parental genotype and regeneration date; GP-derived lines generally showed more of a reduction from the first to the second regeneration dates than did Morex-derived lines.

Comparisons among methods showed a general trend for SMC-derived lines to have the highest yield, and SEC-derived lines to the lowest yield; MEC-derived lines had intermediate yields. This trend was statistically significant most often for GP-derived lines; for Morex, Tukey's analyses showed significance only at the Aberdeen test location in 1998.

Grain Quality

The limited amounts of seed produced by individual plants in the 1997 tests was insufficient for standard analyses of test weight and percentage plump kernels, so 100-kernel-weight was substituted as a similar measure of seed size and density. Analysis of variance indicated significant differences attributable to the method of line production. Regenerated lines tended to have lower 100-kernel-weight than their controls (Table 5), but significant reductions relative to the control were detected only for Golden Promise-derived SEC and SMC lines at the second regeneration date. In general, SEC-derived lines had the lowest, and SMC-derived lines the highest 100-kernel-weights.

Data collected in 1998 showed also that method of line production was a significant source of variability for both test weight and percentage plump kernels. Differences from the controls were detected for test weight (Table 6) and percentage plump kernels (Table 7) only for SEC-derived lines. SMC- and SEC-derived lines were consistently ranked as having the highest and lowest test weights, respectively, when significant differences were detected among the methods. The test weights of MEC-derived lines were intermediate, but most similar to those of SMC-derived lines. This trend was present also for percentage plump kernels, but was less strong.

These data, as did yield data, indicated an interaction between parental genotype and regeneration date, with GP-derived lines showing a stronger trend than Morex-derived lines towards lower performance at the second regeneration date relative to the first.

DISCUSSION

The results of these studies corroborate previous reports of reduced agronomic performance of lines derived from standard embryogenic barley cultures (Bregitzer and Poulson, 1995; Bregitzer et al., 1995; Kihara et al., 1996), and a negative correlation between agronomic

Table 5. Mean 100-seed-weight (in grams) of tissue culture-derived lines and uncultured parental controls grown in 1997 at Aberdeen and Tetonia, ID.

	Golden Promise-derived lines		Morex-derived lines	
	Regeneration date		Regeneration date	
Culture Method	1st	2nd	1st	2nd
Control	3.50	3.50	4.02	4.02
SEC	3.42B†	3.12*B	3.80B	3.93AB
MEC	3.38B	3.36A	3.95AB	3.88B
SMC	3.52A	3.22*B	3.97A	4.02A

* Indicates significant difference (P = 0.05) from control performance, as determined by the Dunnett's test.
† Comparisons between SEC, MEC, and SMC: means followed by the same letter are not significantly different (P = 0.05), as determined by Tukey's means separation procedure.

Table 6. Mean test weight (in kg m^{-3}) of lines derived from three culture methods and uncultured parental controls grown in 1998 at Aberdeen and Tetonia, ID.

	Golden Promise-derived lines		Morex-derived lines	
	Regeneration date		Regeneration date	
Culture Method	1st	2nd	1st	2nd
Control	594†	594	598	598
SEC	589B	567*C	589B	588B
MEC	598A	589B	600A	600A
SMC	602A	604A	602A	601A

* Indicates significant difference (P = 0.05) from control performance, as determined by the Dunnett's test.
† Comparisons between SEC, MEC, and SMC: means followed by the same letter are not significantly different (P = 0.05), as determined by Tukey's means separation procedure.

Table 7. Mean kernel plumpness (%) of lines derived from three culture methods and uncultured parental controls grown in 1998 at Aberdeen and Tetonia, ID.

	Golden Promise-derived lines		Morex-derived lines	
	Regeneration date		Regeneration date	
Culture Method	1st	2nd	1st	2nd
Control	71.4	71.4	86.5	86.5
SEC	68.8A	45.4*C	79.7*B	83.9A
MEC	68.1A	65.3B	85.1A	83.5A
SMC	69.8A	68.5A	84.2A	83.8A

* Indicates significant difference (P = 0.05) from control performance, as determined by the Dunnett's test.
† Comparisons between SEC, MEC, and SMC: means followed by the same letter are not significantly different (P = 0.05), as determined by Tukey's means separation procedure.

performance of regenerated lines and time in culture (Lee et al., 1988). More important, these results indicate that tissue culture systems can be devised which significantly reduce SCV in regenerated plants. Interestingly, our results strongly suggest that the traditional focus on relatively dedifferentiated friable, embryogenic tissues is not ideal from the standpoint of reducing SCV.

In previous work (reviewed in the introduction), we found that MEC and SMC tissues, relative to SEC tissues, support the regeneration of larger numbers of green plants and fewer albino plants. In addition, longevity of regeneration is greatly increased, with MEC and SMC tissues showing minimal losses of regenerability even in cultures older than one year. It is probable that such improvements in regenerability reflect greater genomic stability, a contention supported by the reductions in SCV among lines derived from MEC and SMC tissues observed in this study.

The underlying mechanisms which contribute to the improved regenerability of MEC and SMC tissues, and the reduced SCV in plants recovered from these tissues, is unknown. An important consideration may be differences in the relative growth rates of the three types of tissues. Certainly SCV which is caused or perpetuated by aberrations in the cell division would be expected to be positively correlated to growth rate. Many mechanisms associated with SCV can be related directly or indirectly to cell division, such as mitotic recombination, chromosome breakage and subsequent cytological abnormalities including the activation of transposable elements, and the perpetuation of methylation changes and point mutations.

We do not believe differences in the growth rates of SEC, MEC, and SMC tissues to be great enough to explain the observed differences in agronomic performance of regenerated plants. The SEC and MEC tissues are derived from scutellar tissue of immature embryos, and show great similarities in the processes of initial tissue proliferation, particularly during the first several weeks. The eventual conversion of the MEC tissues to a highly differentiated state does not seem to greatly affect the rate of proliferation. Thus, the direct comparisons based on similar culture age in this study between SEC- and MEC-derived plants are legitimate. SMC tissues, on the other hand, show relatively slower proliferation during the first several weeks of culture initiation, but eventually attain a growth rate comparable to that of the SEC and MEC tissues. Therefore, we cannot claim with certainty that the SMC method is superior to the MEC method with respect to SCV. However, our examination of plants derived from GP cultures that were chronologically older (6 vs. 3 mo), combined with our observations of a correlation of culture age and SCV, does suggest that differences in growth rate are not a major factor in the observed superior agronomic performance of SMC-derived lines. Furthermore, from the standpoint of producing transgenic plants, each of these tissue culture systems can, within the 3-mo culture period studied, be used successfully.

While the mechanisms explaining the observed differences may be unclear, the use of 6-benzyladenine (6-BA) to promote greater levels of tissue differentiation appears to be a critical factor. For both the degree of differentiation and agronomic performance of regenerated plants, the three culture types can be ranked SMC > MEC > SEC. Recent cytological studies on transgenic oat (*Avena sativa* L.) plants clearly show a strong, positive relationship between the use of 6-BA to promote greater differentiation and reductions in cytological aberrations (Choi et al., 2000b).

To our knowledge, this is the first report linking agronomic performance with specific tissue culture systems. However, previous observations of relationships between regenerability characteristics, SCV, and/or the use of 6-BA are consistent with these observations and with previous characterizations of SEC, MEC, and SMC tissues (Cho et al., 1998; Lemaux et al., 1999; Zhang et al., 1999). Bregitzer et al. (1989) reported that globular somatic oat embryos could be diverted into a partially organogenic mode of plant regeneration; furthermore, the use of 6-BA in regeneration medium promoted the recovery of more plants than medium devoid of 6-BA. Armstrong and Phillips (1988) compared cytogenetic variation, pollen sterility, and qualitative phenotypic variation in maize plants derived from type I (organogenic) versus type II (embryogenic) tissues, and concluded that type I culture-derived plants had lower frequencies of abnormalities. Further investigations of the relationships between the level of tissue differentiation and the role of 6-BA as they influence the generation of SCV are in progress. These studies may provide information which may assist the development of less mutagenic tissue culture systems.

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